The interplay between the master transcription factor PU.1 and miR-424 regulates human monocyte/macrophage differentiation

A. Rosa*, M. Ballarino*, A. Sorrentino†, O. Sthandier*, F. G. De Angelis*, M. Marchioni‡, B. Masella†, A. Guarini§, A. Fatica*, C. Peschle†, and I. Bozzoni*‡¶

*Institute Pasteur Cenci-Bolognetti, Department of Genetics and Molecular Biology, ‡Institute of Molecular Biology and Pathology, and §Department of Cellular Biotechnologies and Hematology, University of Rome ''La Sapienza'', Piazzale Aldo Moro 5, 00185 Rome, Italy; and †Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, 00161 Rome, Italy

Edited by Michael Rosbash, Brandeis University, Waltham, MA, and approved October 25, 2007 (received for review July 25, 2007)

We describe a pathway by which the master transcription factor PU.1 regulates human monocyte/macrophage differentiation. This includes miR-424 and the transcriptional factor NFI-A. We show that PU.1 and these two components are interlinked in a finely tuned temporal and regulatory circuitry: PU.1 activates the transcription of miR-424, and this up-regulation is involved in stimulating monocyte differentiation through miR-424-dependent translational repression of NFI-A. In turn, the decrease in NFI-A levels is important for the activation of differentiation-specific genes such as *M-CSFr*. In line with these data, both RNAi against NFI-A and ectopic expression of miR-424 in precursor cells enhance monocytic differentiation, whereas the ectopic expression of NFI-A has an opposite effect. The interplay among these three components was demonstrated in myeloid cell lines as well as in human CD34+ differentiation. These data point to the important role of miR-424 and NFI-A in controlling the monocyte/macrophage differentiation program.

hematopoietic differentiation | microRNA | NFI-A | monocytopoiesis

Lineage specification of hematopoietic multipotential progenitors is a multistep process controlled by a complex system of interacting transcription factors (1). Numerous data indicate that several such relevant factors are already expressed in the hematopoietic stem cell (HSC), although at a low level (2, 3), establishing the so-called transcriptional priming that would reflect the developmental potency of the multilineage precursor.

Some of these factors, such as SCL/Tal1 and AML1, are multipotent, and their depletion affects the entire blood cell differentiation. On the other hand, other factors have a lineage-specific expression such as GATA1, C/EBPα, and PU.1. In the case of myeloid differentiation, the relative levels of PU.1 and C/EBPα have been suggested to regulate monocytic vs. granulocytic cell fate choice (4, 5). Interestingly, these factors have functional interconnections, because C/EBPα and GATA1 can inhibit PU.1 function (4, 6), whereas increases in PU.1 lead to inhibition of GATA1 (7). Interestingly, PU.1 not only promotes its own transcription (8) but also prevents the activation of genes involved in alternative pathways (1, 9, 10). Different models involving either antagonistic cross-regulation (1, 9) or cooperative interplay (10) between primary lineage-determining transcriptional factors have been proposed for explaining how the initiation and maintenance of lineage differentiation can be achieved.

In addition to the primary role of transcription factors, a family of gene expression regulators, the microRNAs (miRNAs), has been recently shown to play a crucial role in hematopoietic lineage differentiation (11, 12). The first case was described in mice; ectopic expression of miR-181 in hematopoietic progenitor cells led to an increased fraction of B-lymphoid cells in both tissue-culture differentiation assays and adult mice (13). Similarly, miR-223 was shown to increase upon granulocytic differentiation and to promote maturation of promyelocytic precursors into granulocytes in humans (14). In other studies, the down-regulation of miR-221 and -222 was shown to be important for erythropoietic differentiation of human cord blood CD34+ progenitors (15), and the down-regulation of miR17–5p, 20a, and 106a was required for monocytopoiesis (16).

The targets of miRNA translational repression are often represented by transcription factors playing crucial roles in differentiation. This is the case for MAFB and HOXA1, factors required for terminal commitment of megakaryocytic cells, that were shown to be the targets of miR-130 and -10, respectively (17). In some cases, specific miRNAs and their transcriptional activators happen to be in regulatory feedback loops in which they control each other (14, 16). These loops may represent regulatory mechanisms allowing relatively small variations in miRNA concentration to induce drastic changes in the cellular transcriptional patterns.

In the present work, we describe a pathway by which the up-regulation of the lineage-specific factor PU.1 controls human monocyte/macrophage differentiation. This includes the activation of miR-424 that synergizes with PU.1 for the activation of terminal differentiation genes through the repression of NFI-A.

Results

miR-424 Up-Regulation Is Associated with Human Monocyte/Macrophage Differentiation. miR-424 was previously identified among the miRNAs up-regulated by TPA-induced monocyte/macrophage differentiation of the human HL60 leukemia cell line (18). To study the role of miR-424 in this differentiation program, we first analyzed its levels in human cord blood CD34+ cells (19) induced to differentiate to the monocyte/macrophage-specific unilineage. Fig. 1*A* shows an increase from day 6 to day 12 (reaching a 7-fold accumulation with respect to progenitor cells), indicating that miR-424 up-regulation occurs in normal cells upon induction to monocyte/macrophage-specific differentiation. miR-424 expression was also tested in cells freshly taken from an Acute Promyelocytic Leukemia (APL) patient (FAB subtype-M3) induced to differentiate *in vitro* to monocytes/macrophages. Fig. 1*B* indicates that also in this case, miR-424 is up-regulated (4- to 5-fold) in response to the differentiation stimulus.

The NB4 cell line, derived from the bone marrow of an APL patient, is a genuine human promyelocytic cell line (FAB subtype - M3; ref. 20) that has provided a powerful *in vitro* model system of

Author contributions: A.R. and M.B. contributed equally to this work; I.B. designed research; A.R., M.B., A.S., O.S., F.G.D.A., M.M., and A.F. performed research; A.S., O.S., F.G.D.A., B.M., A.G., and C.P. contributed new reagents/analytic tools; A.R., M.B., A.F., C.P., and I.B. analyzed data; and I.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¶To whom correspondence should be addressed. E-mail: irene.bozzoni@uniroma1.it.

This article contains supporting information online at www.pnas.org/cgi/content/full/0706963104/DC1.

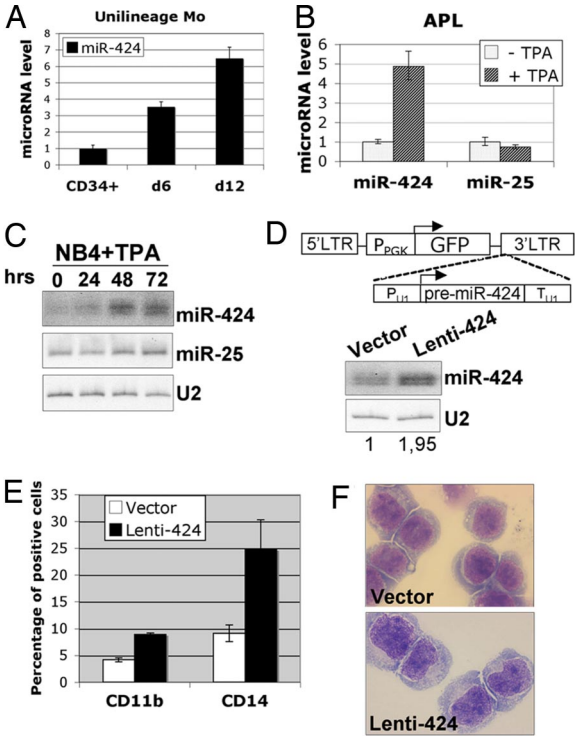

Fig. 1. Role of miR-424 in monocyte/macrophage differentiation. miRNA TaqMan microRNA assays (Applied Biosystem) on total RNA from: (*A*) CD34+ cells induced to differentiate to the monocytic lineage (samples taken at the indicated days) and (*B*) cells from an APL patient, before (−TPA) and 48 h after TPA treatment (+TPA). miR-25, whose levels do not change in differentiation, is used as control. (*C*) Ten micrograms of RNA, from untreated NB4 cells (lanes 0) or from the same cells treated with TPA for the indicated times, was analyzed by Northern blot with the probes indicated on the side of each image. Endogenous spliceosomal U2 snRNA and miR-25 were used as loading controls. (*D Upper*) Schematic representation of the lentiviral construct for miR-424 expression (Lenti-424); (*Lower*) Northern blot analysis of 10 μg of total RNA extracted from NB4 cells infected with the empty vector (lane Vector) or with Lenti-424 (lane Lenti-424) and incubated for 48 h. miR-424 signals were normalized for U2 snRNA hybridization and the values, expressed as fractions with respect to mock-treated cells (Vector), are indicated below each lane. (*E*) Percentage of CD11b- or CD14-positive cells in NB4 cells ectopically expressing either the empty vector or Lenti-424. (*F*) Morphological analysis of NB4 cells 7 days after infection with the empty vector or with Lenti-424. In all histograms, the values represent the means ± SEM from triplicates.

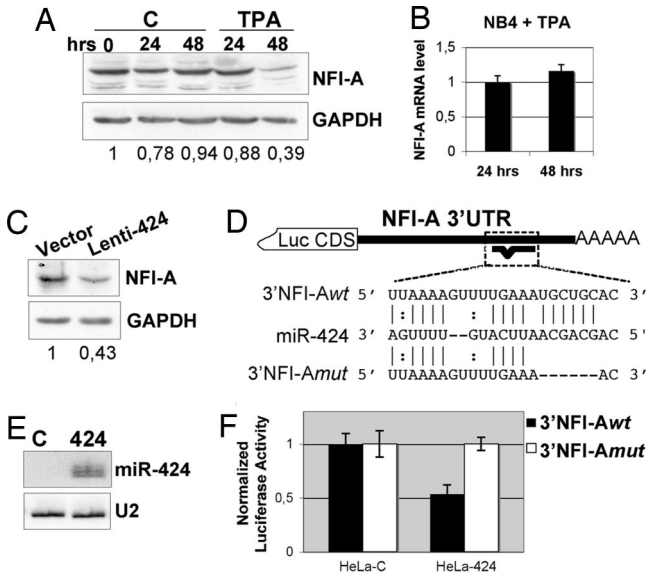

Fig. 2. NFI-A is a miR-424 target. (*A*) Total proteins, from NB4 cells grown for the indicated hours in the absence (lanes C) or presence of TPA (lanes TPA), were analyzed by Western blot with anti-NFI-A antibodies. Signals were normalized for GAPDH and the values, expressed as fractions with respect to time 0, are indicated below. (*B*) qRT-PCR analysis of NFI-A mRNA levels in NB4 cells treated with TPA for the indicated times. The histograms represent the means ± SEM from triplicates. (*C*) Western blot analysis of proteins extracted from cells infected with the empty vector of with the Lenti-424. Signals were normalized for GAPDH, and the values, expressed as fractions with respect to mock-treated cells (Vector), are indicated below. (*D*) Schematic representation of the constructs used in the luciferase assay. The sequences shown below indicate: the putative miR-424 target site on the wild-type 3′UTR (construct 3′NFI-A*wt*), its mutated derivative (construct 3′NFI-A*mut*), and the pairing regions of miR-424. (*E*) Northern blot analysis of HeLa cells infected with the empty vector (lane C) or with Lenti-424 (lane 424). (*F*) Cells were infected with Lenti-424 (HeLa-424) or with the control vector (HeLa-C) and then transfected with either 3′NFI-A*wt* (black boxes) or 3′NFI-A*mut* (white boxes). Renilla luciferase activity was normalized to Firefly luciferase activity, and then the forward luciferase-containing constructs were normalized to the mutated controls. Error bars represent the SEM from triplicates.

differentiation: whereas all-trans retinoic acid (ATRA) treatment induces differentiation to morphologically and functionally mature granulocytes, TPA induces a monocyte/macrophage phenotype already after 48 h of treatment. Fig. 1*C* shows that also in TPA-treated NB4 cells miR-424 is up-regulated, reaching a 5-fold accumulation after 48–72 h (Fig. 1*C*). Similar up-regulation was found in the HL60 cell line (supporting information (SI) Fig. 6), derived from a patient with acute promyeloblastic leukemia (21) and consisting predominantly of promyeloblasts (FAB subtype -M2). No accumulation was detected in mature B and T lymphocytes (SI Fig. 6). In addition, no up-regulation was detected upon RA treatment of NB4 cells (SI Fig. 6).

The role of miR-424 in monocyte/macrophage differentiation was analyzed by its ectopic expression in promyelocytic cells. Overexpression of miR-424 was accomplished by infecting NB4 cells with a lentiviral vector containing a cassette in which the pri-miRNA is cloned under the U1snRNA regulatory regions (Lenti-424, Fig. 1*D*). Forty-eight hours after infection, the miR-424 levels resulted in a 2-fold increase compared with mock-transduced cells (Fig. 1*D*). The expression levels of two markers of monocytic/macrophage differentiation were measured by flow cytometry: CD11b is a surface marker of granulocytic and monocytic cell differentiation, whereas CD14 specifically recognizes cells of the monocytic lineage. Fig. 1*E* shows that CD11b-positive cells doubled, whereas those positive for CD14 reached a 3-fold increase. Notably, the ectopic expression of miR-424 also induced morphological changes in NB4 cells, such as a bluish-gray cytoplasm and a saddle-shaped nucleus, consistent with their maturation into monocytes (Fig. 1*F*).

Altogether, these data indicate that the artificial increase of miR-424 levels is able, in the absence of TPA, to induce differentiation of promyelocytic precursors into the monocyte/macrophage lineage.

NFI-A Is a Bona Fide Target of miR-424. Among the hundreds of predicted regulatory targets of miR-424 (22), we noticed the transcription factor NFI-A, a protein previously shown to be down-regulated by miR-223 during granulopoiesis (14). Interestingly, a University of California, Santa Cruz (UCSC), genome browser analysis (www.genome.ucsc.edu) revealed that the putative target site for miR-424 in the NFI-A 3′UTR is highly conserved among mammals as well as miR-424 itself (miR-322 in mouse and rat; SI Fig. 7). After TPA-induced monocytic differentiation, we observed a strong decrease in the NFI-A levels (Fig. 2*A*) without a concomitant decrease of the mRNA levels (Fig. 2*B*). A similar

decrease in the protein levels was detected also when miR-424 was ectopically expressed in the absence of TPA (Fig. 2*C*). These data suggested a functional link between miR-424 and NFI-A accumulation.

To prove that the NFI-A mRNA is a miR-424 target, the 3′ UTR of NFI-A was inserted downstream of a luciferase ORF (3′NFI-A*wt*; Fig. 2*D*). A construct containing a mutated sequence of the miRNA-binding site, 3′NFI-A*mut*, was also produced as a control. The different luciferase constructs were cotransfected into HeLa cells previously infected with Lenti-424 or with the control vector. The efficient expression of miR-424 in these cells was analyzed by Northern blot (Fig. 2*E*). The measurement of luciferase activity indicated a specific repression by miR-424 on the wild-type substrate and no effect when the target site was mutated (Fig. 2*F*). The decrease in luciferase activity was similar to the translational repressor effect observed for other miRNAs on their substrates (14, 23). Altogether, these data demonstrated that NFI-A is a target of miR-424.

NFI-A Counteracts Monocytic Differentiation. RNAi against NFI-A was performed to analyze its role in monocytic differentiation. The infection of NB4 cells with a lentiviral construct expressing siRNAs against the NFI-A mRNA resulted in a strong decrease in the levels of the endogenous protein (Fig. 3*A*). In the presence of TPA, this resulted in a strong increase of both CD11b and CD14 surface antigens when compared with mock-treated cells (Fig. 3*B*). Interestingly, in the same cells, the down-regulation of NFI-A resulted in increased mRNA levels of the monocyte terminal differentiation gene M-CSFr (Fig. 3*C*), whereas no effect was observed for the granulocyte-specific marker G-CSFr (data not shown). On the contrary, the lentiviral-mediated overexpression of a NFI-A derivative with a 3′UTR devoid of miRNA target sites (Fig. 3*D*) counteracted the TPA-induced monocytic differentiation, as evaluated by the reduced levels of the CD11b and CD14 antigens (Fig. 3 *F* and *G*), the M-CSFr mRNA expression levels (Fig. 3*H*) and morphological analysis (Fig. 3*E*). Finally, the treatment of NB4 cells with LNA oligonucleotides against miR-424 resulted in increase of NFI-A levels as well as in reduction of the differentiation markers (SI Fig. 8).

These results show that NFI-A down-regulation is important for monocytic differentiation, therefore indicating that one of the pathway by which miR-424 promotes monocytopoiesis is through NFI-A repression.

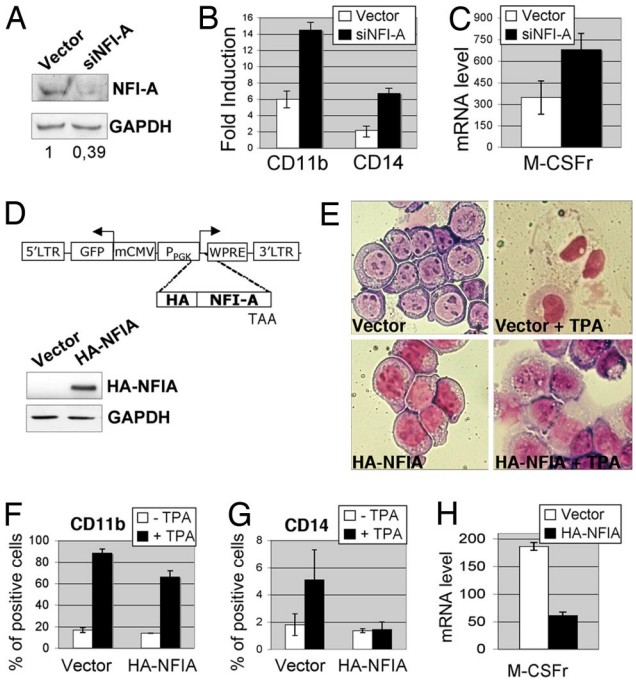

Fig. 3. NFI-A knockdown and overexpression. NB4 cells were infected with the empty lentivector (Vector) or with a lentiviral construct expressing siRNAs against NFI-A (siNFI-A). (*A*) Proteins were extracted 48 h after infection and 50 μg analyzed by Western blot with anti-NFI-A and control anti-GAPDH antibodies. Signals were normalized for GAPDH and the values, expressed as fractions with respect to mock-treated cells (Vector), are indicated below each lane. (*B*) CD11b- or CD14-positive cells were analyzed by FACS; the values indicate the fold induction of TPA-induced positive vs. untreated cells. (*C*) Expression levels of M-CSFr mRNA measured by qRT-PCR in NB4 cells infected with the siNFI-A lentiviral construct or with an empty vector. The values indicate the fold induction of TPA-induced cells vs. untreated ones. (*D*) Schematic representation of Lenti-HA-NFIA and Western analysis with an anti-HA antibody of its ectopic expression in NB4 cells. (*E–H*) NB4 cells were infected with the empty lentiviral vector (Vector) or with the Lenti-HA-NFIA. After the infection, half of the culture was treated with (+TPA) and half without TPA (−TPA). Cells were analyzed by FACS for CD11b (*F*) and CD14 (*G*) expression, by qRT-PCR for M-CSFr expression (*H*), and by Wright–Giemsa staining for morphology (*E*). For each image, the histograms represent the means ± SEM from triplicates.

PU.1 Is Responsible for miR-424 Activation. Two transcriptional start sites for the pri-miR-424 were identified, by 5′ RACE on RNA from cells depleted by RNAi for the Drosha enzyme, at 256 and 227 nucleotides upstream of the premiRNA 5′ end (Fig. 4*A*; F. Pagano, personal communication). The region upstream to these sites was analyzed with the ChIP Mapper software (24) for hematopoietic transcription factor-binding sites, and one canonical PU.1-binding site (25) was found at position −1681 −1671. Interestingly, a UCSC genome browser analysis revealed that this site is embedded in a region highly conserved in mammals (SI Fig. 9). PU.1 is a key factor for the commitment of hematopoietic cells to the monocytic lineage (1) and, in NB4 cells (Fig. 4*B*) as well as in HL60 (SI Fig. 6*D*), undergoes activation upon TPA treatment. The protein reaches a maximum level at 24 h and decreases afterward. Interestingly, the up-regulation of PU.1 precedes miR-424 activation (compare Figs. 1*C* and 4*B* and SI Fig. 6 *B* and *D*).

To investigate whether PU.1 physically interacts *in vivo* with the miR-424 promoter, we performed a ChIP assay on uninduced and TPA-treated cells. DNA from the PU.1 immunoprecipitates was amplified with a couple of PCR primers (prom/1) located in the promoter region surrounding the putative PU.1-binding site (Fig. 4*A*). Specific PU.1 interaction was detected after TPA induction (Fig. 4*C*, miR-424). The specificity of PU.1 occupancy in the miR-424 promoter was demonstrated by the absence of immunoprecipitation with oligos corresponding to an unrelated genomic region (Fig. 4*C*, UR). As a positive control, PU.1 immunoprecipitation was tested on the high-affinity PU.1-binding site present in the −14-kb URE3′ region of the PU.1 promoter (26). Fig. 4*C* (URE3′) shows that the PU.1 factor interacts efficiently with this region, and that its binding is induced by TPA, as observed for miR-424. Moreover, EMSA performed with three oligos, corresponding to regions prom/1, prom/2 (an unrelated miR-424 upstream region), and URE3′, and nuclear extracts from cells induced with TPA for 24 h, showed that a specific shift occurs on the prom/1 region that corresponds to the one obtained with the positive URE3′ control (SI Fig. 10).

These results confirmed that the PU.1 factor can bind *in vitro* and *in vivo* to the miR-424 promoter.

To analyze the correlation between PU.1 and miR-424 transcription, we also analyzed the miRNA expression levels in cells where PU.1 was knocked-down by RNAi. Fig. 4*D* shows that a 50% reduction in the PU.1 levels were obtained in cells infected with a lentiviral construct expressing siRNAs against PU.1 (Lenti-siPU.1). In these cells, miR-424 does not undergo up-regulation after the TPA-treatment (Fig. 4*E*, compare lanes TPA+).

Promoter fusion constructs were used to further correlate the binding of PU.1 with miR-424 transcriptional activity. Two thou-

CELL BIOLOGY

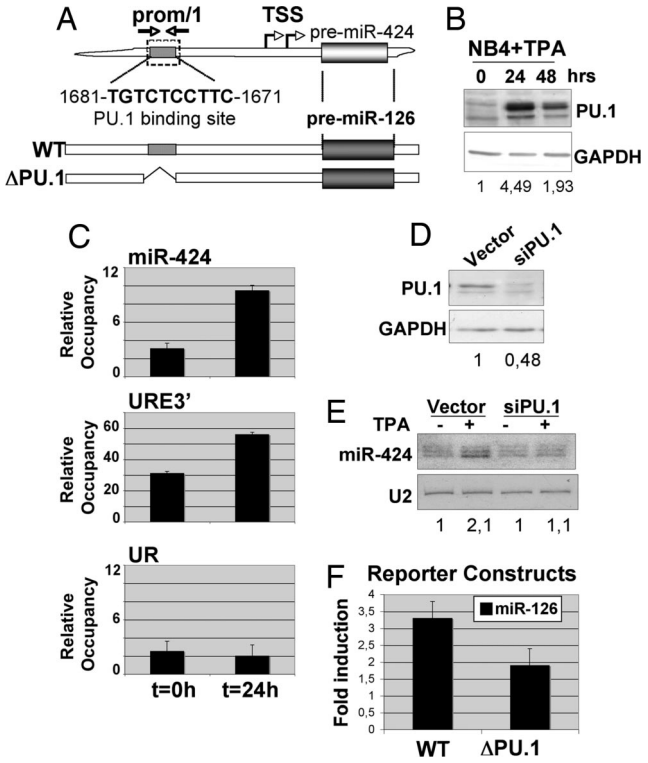

Fig. 4. PU.1 binds the miR-424 promoter. (*A*) Schematic representation of the miR-424 genomic region and of the constructs containing the wild-type (WT) or mutant (ΔPU.1) miR-424 promoter fused to the premiR-126 coding region. The transcriptional start sites (TSS) are indicated by open arrows. The sequence and location of the PU.1-binding site are indicated by the dashed box, whereas the prom/1 arrows point to the regions amplified by qPCR in the ChIP experiments. (*B*) NB4 cells were treated with TPA for the indicated times. Fifty micrograms of total protein was analyzed by Western blot with anti-PU.1 antibody. Signals were normalized for GAPDH, and the values, expressed as fractions with respect to time 0, are indicated below. (*C*) Chromatin from cells at different times of induction was immunoprecipitated with anti-PU.1 antibodies, and the recovered DNA was submitted to qPCR with prom/1 oligos (miR-424). Oligonucleotides corresponding to the −14-kb URE3′ region of the PU.1 promoter were used as positive control (URE3′). An unrelated genomic region (UR) was amplified as a negative control (UR). The histograms represent the means ± SEM from three independent experiments. (*D*) NB4 cells were infected with a lentiviral construct expressing siRNAs against PU.1 (siPU.1) or with an empty vector. Fifty micrograms of proteins was analyzed by Western blot analysis with anti-PU.1. Signals were normalized for GAPDH, and the values, expressed as fractions with respect to mock-treated cells (Vector), are indicated below. (*E*) Northern blot analysis of RNA from control cells (lanes −) or cells treated with TPA for 48 h (lanes +) and either infected with an empty vector (lanes Vector) or with Lenti-siPU.1 (lanes siPU.1). miR-424 signals were normalized for U2 snRNA hybridization, and the values, expressed as fractions with respect to TPA-minus samples, are indicated below. (*F*) NB4 cells were electroporated with WT or ΔPU.1 constructs (*A*), and the amount of miR-126 was measured with the Applied Biosystems TaqMan MicroRNA Assay. The values were normalized against the U6 snRNA. The histograms represent the means ± SEM from triplicates.

sand base pairs of the miR-424 upstream region, or a mutated version deleted of the PU.1-binding site, were fused to the coding region of the premiR-126 (see schematic representation in Fig. 4*A*). After transfection in NB4 cells, half of the culture was treated with TPA for 48 h, and half was maintained without the inducer for the same time. Fig. 4*F* shows that, compared with the WT promoter, the ΔPU.1 construct produced half the levels of miR-126 transcription upon TPA treatment. In conclusion, these experiments showed that PU.1 interacts *in vivo* with the miR-424 promoter and is necessary for at least 50% up-regulation of miR-424 upon TPA induction.

Role of the PU.1/miR-424/NFI-A Circuitry in Normal Hematopoiesis. The expression profiles of miR-424, PU.1, and NFIA were analyzed during normal hematopoiesis, specifically in the monocytic/macrophage unilineage differentiation of human cord blood CD34+ precursor cells. In this *in vitro* system, >95% purified human cord blood CD34+ progenitors undergo a gradual, synchronized, and selective differentiation maturation through the monocyte/macrophage lineage (19). The cultures were monitored along a 2-week period; differentiation was evaluated by morphological and immunophenotype analysis, particularly for the appearance and rise of $CD14^+$ cells. The percentage of $CD14^+$ cells is shown in Fig. 5*A Top*. The miR-424 expression profile indicated this miRNA progressively accumulates during differentiation, paralleling the CD14 marker (Fig. 5*A Middle*). Western analysis (Fig. 5*A Bottom*) showed that the PU.1 protein is produced very early after induction and is persistently expressed, perfectly paralleling miR-424 accumulation. At variance with PU.1 and miR-424, the NFI-A protein is already present in $CD34^+$ cells, and its levels strongly decrease at day 4 when the levels of miR-424 have already doubled. This is in agreement with what was observed in the cell lines: in a 24-h interval after TPA treatment, whereas miR-424 doubles, NFI-A undergoes almost a 3-fold reduction (compare 24 and 48 h in Figs. 1 and 2).

To prove the physiological relevance of the circuitry described in cell lines, $CD34^+$ progenitor cells induced to differentiate toward the monocyte/macrophage lineage were infected with the lentiviral constructs to knockdown PU.1 (siPU.1) or to overexpress either miR-424 (424) or the miR-resistant form of NFI-A (HA-NFIA). After GFP sorting, the differentiation status of the transduced cells was assessed by cell growth, CD14 expression, and morphology. When compared with cells infected with the empty vector, the overexpression of miR-424 resulted in an increase of the differentiation parameters (Fig. 5*B*). On the contrary, both the enforced expression of the miR-resistant form of NFI-A or the knockdown of PU.1 counteracted differentiation (Fig. 5*C*). To have a CD14-independent evaluation of the effect on differentiation of siPU.1 and HA-NFI-A, we have quantified the morphological data, proving that the effect of both constructs on counteracting differentiation is relevant and similar (50% reduction) in both cases (*Right*).

In conclusion, these experiments demonstrated that the altered expression of PU.1, miR-424, and NFI-A has similar outcomes on the monocyte/macrophage differentiation program of promyelocytic cell lines as well as human CD34+ progenitors.

Discussion

In this study, we identified a PU.1-dependent regulatory pathway, required to commit promyelocytic blasts to the monocyte/macrophage lineage, which consists of the specific up-regulation of miR-424. The PU.1 factor was shown to interact with the miR-424 promoter and to be responsible for at least 50% of its activation upon TPA treatment. miR-424 resulted in an important effector for the commitment to differentiation, because its ectopic expression in the myeloid precursors was shown to promote monocytic differentiation in the absence of the inducer. Therefore, miR-424 appears to have a high hierarchical position among the factors regulating this hematopoietic lineage.

Among the numerous putative targets of miR-424, the NFI-A mRNA was validated as a true functional one. RNAi against NFI-A in TPA-treated promyelocytic cells enhanced monocytic differentiation, whereas the ectopic expression of a miR-resistant form of NFI-A or the LNA-mediated depletion of miR-424 produced the opposite effect. These results indicated that NFI-A down-regulation is indeed required for progression to monocytic differentiation, similarly to what is shown in granulopoiesis (14). Therefore, NFI-A appears to have the common function of counteracting differentiation in both myeloid lineages. Notably, miR-223 and -424, both repressors of NFI-A, are activated by the master regulatory factors of the corresponding lineages, C/EBPα and PU.1,

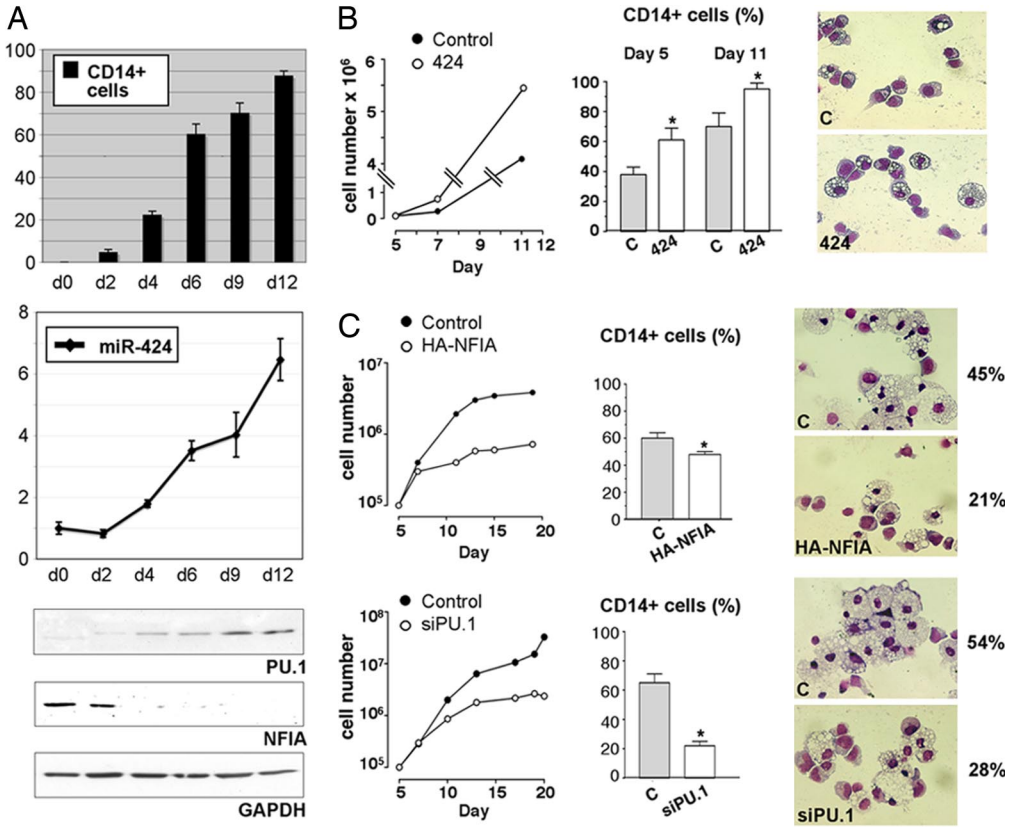

Fig. 5. Role of PU.1, miR-424, and NFI-A in normal hematopoiesis. (*A*) Monocyte/macrophage differentiation of human CD34+ cells. Samples were collected at the indicated days after induction. (*Top*) FACS analysis of $CD14^+$ cells; (*Middle*) TaqMan microRNA assays of miR-424 (Applied Biosystems). The values are normalized with the U6 snRNA, and error bars represent standard errors from triplicates. (*Bottom*) Western blot analysis on total proteins (15 μg) with antibodies against the indicated proteins. (*B*) CD34+ cells, induced to differentiate toward the monocyte/macrophage lineage, were infected with Lenti-424 and sorted for the GFP marker carried by the vector. Growth in hematopoietic progenitor cell–monocyte (HPC M) medium. (*Left*), CD14 expression (*Center*), and morphological analysis at day 7 (*Right*) are shown. (*C*) CD34+ cells, induced to differentiate toward the monocyte/macrophage lineage, were infected with Lenti-HA-NFIA (*Upper*) or Lenti-siPU.1 (*Lower*) and sorted for the GFP marker carried by the vector. (*Left*) Growth in HPC M culture; (*Center*) CD14+ expression at day 10 (*Right*) and day 8 (*Lower*). (*Right*) Morphological analyses at day 10; representative fields are shown together with the percentage of mature cells. In all histograms, the values represent the means ± SEM from three separate experiments. *, $P < 0.01$ compared with control.

respectively. These conclusions were subsequently validated in a well established system for unilineage differentiation/maturation of $CD34^+$ hematopoietic progenitors (19). In unilineage monocyte/macrophage culture, miR-424 activation paralleled the expression profile of PU.1, whereas NFI-A displayed an inverse correlation with respect to miR-424, similarly to what is observed in APL cell lines. Moreover, experiments of PU.1 knockdown and miR-424 and NFI-A ectopic expression confirmed that the PU.1/miR-424/NFI-A circuitry identified in cell lines indeed regulates normal monocytic differentiation.

In conclusion, our data show that miR-424 synergizes with PU.1 in inducing the gene expression pattern required for monocyte/macrophage differentiation and in establishing strong transcriptional commitments. In addition, they point to an important function of NFI-A in the differentiation commitment of two myeloid-specific pathways (granulocyte and monocyte/macrophage) and to the role of lineage-specific miRNAs in controlling the levels of this factor.

Experimental Procedures

Reagents. TPA and RA were purchased from Sigma and used for the indicated times after concentration (TPA, 1.6 nM and RA, 1 μM).

Cell Cultures. Primary cells were obtained at the time of diagnosis from the bone marrow of a patient with acute promyelocytic leukemia carrying the PML/RARα fusion (FAB classification, M3). Leukemic cells, isolated on Ficoll–Hypaque density gradients, and NB4 and HL60 cell lines were cultured as described (14). Monocytic (Mo) unilineage cultures were performed according to (19)

Constructs. The psiU-424 plasmid was generated by cloning a fragment containing the premiR-424 (from −120 to +160 bp relative to the 5′-end of mature miR-424) into the psiUx plasmid (27). The constructs for RNAi were also raised in psiUx; they transcribe a short hairpin that produces the accumulation of siRNAs against NFI-A (14) or against PU.1 target sequence (5′-GTCCGTATGTAAATCAGATCT-3′). The psiU-derived expression cassettes were subcloned into the 3′-LTR of the lentiviral vector pRRLcPPT.hPGK.EGFP.WPRE, as described (14). The miR-resistant form of NFI-A was obtained by RT-PCR amplification of its coding region, devoid of the 3′ UTR, with oligos: forward: 5′-ATGTATTCTCCGCTCTGTCT-3′ and reverse: 5′-TTATCCCAGGTACCAGGACT-3′. NFI-A was HA-tagged at the N terminus and cloned in the EcoRV and SalI sites of the bidirectional pCCL.sin.cPPT.PGK.mCMV.GFP.WPRE lentiviral vector (28). Infective particles were produced according to ref. 29.

For promoter analysis, a DNA fragment extending from position −2,000 to −25 bp (WT) relative to the pre-miR-424 was cloned upstream to a reporter pri-miR-126 gene (14). The ΔPU.1 clone has a deletion corresponding to the PU.1 consensus site. Twenty micrograms of each construct were transfected by electroporation into NB4 cells. After 3 hours, cells were divided in two and one aliquot treated with TPA for 48 h. Total RNA was analyzed by the TaqMan MicroRNA Assay (Applied Biosystems).

miRNA Knockdown. Locked nucleic acid (LNA) oligonucleotides, FITC-labeled by the manufacturer (Exiqon), were individually transfected by Lipofectamine 2000 (Invitrogen) into NB4 cells as described (14).

RNA Analysis. Total RNA, extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, was analyzed by Northern blot as described (14). The following oligonucleotides were used as probes: α-223, 5′-GGGGTATTTGACAAACTGACA-3′; α-424, 5′-TTCAAAACATGAATTGCTGCTG-3′;

α-25, 5′-CAGACCGAGACAAGTGCAATG-3′. The endogenous U2 snRNA was detected with oligo U2R, 5′-GGGTGCACCGTTCCTGGAGGTAC-3′.

Where indicated, miRNA quantification was performed by real-time PCR in ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Delta-delta Ct values were normalized with those obtained from the amplification of the endogenous U6 snRNA. All reactions were performed in triplicate.

mRNA quantification was performed by real-time PCR with iTaq SYBR green supermix (BioRad), according to the manufacturer's instructions. The following oligonucleotides were used: M-CSFr up, 5′-TCCAAAACACGGGGACCTATC-3′ and down, 5′-TCCTCGAACACGACCACCT-3′. The NFI-A transcript was detected with TaqMan oligonucleotides HS 00325656.m1 (Applied Biosystems). Delta-delta Ct values were normalized with those obtained from the amplification of the endogenous GAPDH mRNA with Quantitect Primer Assay (Qiagen). All reactions were performed in triplicate.

Immunoblot Analysis. Fifty micrograms of proteins were fractionated by electrophoresis on 10% SDS polyacrylamide gel, electroblotted onto nitrocellulose membrane (Protran, S&S), and reacted with anti-NFI-A (Abnova), anti-PU.1 (Cell Signaling Technology), anti-GAPDH (Abcam), or anti-HA (Santa Cruz Biotechnology) antibodies. Immunoreactivity was determined by using the ECL method (Amersham) according to the manufacturer's instructions.

Chromatin Immunoprecipitation Assay. DNA/protein cross-linking was obtained by incubating the cells for 20 min at 37°C in 1% formaldehyde. After sonication, chromatin was immunoprecipitated overnight with 10 μl of anti-PU.1 antibody (Cell Signaling Technology).

Real-time PCRs of genomic regions containing the putative PU.1-binding site were performed in triplicate by using iTaq SYBR green supermix (BioRad) with oligos prom/1a (5′-TACATCGTGTGTTTGGGGTG-3′) and prom/1b (5′-ACGCCTCTTCCTCTGTTCATAC-3′). Control amplifications were performed with oligos URE3′a (5′-TGGCTCTGGTCTCAACTCTG-3′) and URE3′b (5′-GCAGGAAAGAGGAAGGC-3′) and URa (5′-CCAGCTGATTGAGAATGCAGA-3′) and URb (5′-AAGGACACTAGGTGGTTGAGA-3′). The relative occupancy of the immunoprecipitated factor at a locus is estimated by using the comparative threshold method (30): 2^(Ctmock–Ctspecific), where Ctmock and Ctspecific are mean threshold cycles of PCR done in triplicate on DNA samples from mock and specific immunoprecipitations.

EMSA for *in Vitro* DNA Binding. Nuclear extract from NB4 cells treated with TPA, 1.6 nM, for 24 h was prepared as described (25). The following oligonucleotides were annealed, labeled with [α-P^{32}]ATP by the use of Klenow enzyme, and incubated with 30 μg of NB4 extracts in 10 mM Hepes (pH 7.8), 50 mM KCl, 1 mM DTT, 1 mM EDTA, 0.5 μg of polidIdC, and 5% glycerol for 20 min at 0°C.: prom/1fw (5′-GATCATGAACAGAGGAAGAGGCGTATTC-3′), prom/1rev (5′-GATCGAATACGCCTCTTCCTCTGTTCAT-3′); prom/2fw (5′-GATCGCTTGAAACAGGAAGGAGCGACTT-3′), prom/2rev (5′-GATCAAGTCGCTCCTTCCTGTTTCAAGC-3′); URE3′fw (5′-GATCCGGCCTTGCTGCTGCCGATGTGGA-3′), URE3′rev (5′-GATCTCCACATCGGCAGCAGCAAGGCCGG-3′). Reaction mixtures were separated with 6% polyacrylamide gels in 0.5× TBE buffer at 4°C.

Luciferase Assays. The pRL-TK-3′NFI plasmid (14) was used to generate the 3′NFI-A*mut* plasmid containing a mutated site for miR-424. Five hundred nanograms of pRL-TK derivative (Rr-luc) and 50 ng of pGL3 control vector (Pp-luc; Promega) were cotransfected in HeLa cells previously infected with Lenti-424 or an empty vector. Cells were harvested 24 h posttransfection and assayed with Dual Luciferase Assay (Promega) according to the manufacturer's instructions.

Immunophenotyping and Morphological Analysis. Cell differentiation was evaluated by immunofluorescence staining by using primary mouse anti-human CD11b and mouse anti-human CD14 cell surface myeloid-specific antigens (eBiosciences) and a secondary anti-mouse IgG R-phycoerythrin conjugate (Immunological Sciences). A minimum of 10,000 events were collected for each sample by a FACScan flow cytometer (Becton Dickinson) by using CellFit software (BD Biosciences) for data acquisition and analysis. Morphology was evaluated in conventional light-field microscopy of Wright–Giemsa-stained cytospins at a magnification of ×400.

We thank Dr. M. Cavaldesi for FACS analysis and Prof. A. Santoni, Dr. M. Cippitelli, and Dr. D. Milana (Department of Experimental Medicine, "La Sapienza," University of Rome) for B and T cell-specific RNAs. We also thank Dr. L. Naldini (University Vita Salute "San Raffaele," Milan) for providing lentiviral vectors, T. Incitti and M. L. De Marchis for helping with plasmid construction, M. Arceci for technical assistance, and A. Zito for graphics. This work was partially supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC) and AIRC-ROC, Sixth Research Framework Program of the European Union; Project RIGHT (Grant LSHB-CT-2004 005276), and SIROCCO (Grant LSHG-CT-2006–037900); Ministero dell'Universitá e della Ricerca Scientifica e Technologica (Grants FIRB-p.n. RBNE015MPB and RBNE01KXC9); Progetti di Ricerca di Interesse Nazionale and "Centro di Eccellenza Biologia e Medicina Molecolare;" and the Italy–US Oncology Program on MicroRNAs (C.P.).

1. Tenen DG (2003) *Nat Rev Cancer* 3:89–101.
2. Hu M, Krause D, Greaves M, Sharkis S, Dexter M, Heyworth C, Enver T (1997) *Genes Dev* 11:774–785.
3. Akashi K, He X, Chen J, Iwasaki H, Niu C, Steenhard B, Zhang J, Haug J, Li L (2003) *Blood* 101:383–389.
4. Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG (1998) *Mol Cell Biol* 18:4301–4314.
5. Scott EW, Simon MC, Anastasi J, Singh H (1994) *Science* 265:1573–1577.
6. Reddy VA, Iwama A, Iotzova G, Schulz M, Elsasser A, Vangala RK, Tenen DG, Hiddemann W, Behre G (2002) *Blood* 100:483–490.
7. Zhang P, Zhang X, Iwama A, Yu C, Smith KA, Mueller BU, Narravula S, Torbett BE, Orkin SH, Tenen DG (2000) *Blood* 96:2641–2648.
8. Chen H, Ray-Gallet D, Zhang P, Hetherington CJ, Gonzalez DA, Zhang DE, Moreau-Gachelin F, Tenen DG (1995) *Oncogene* 11:1549–1560.
9. Orkin SH (2000) *Nat Rev Genet* 1:57–64.
10. Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, Sciammas R, Gantner BN, Dinner AR, Singh H (2006) *Cell* 126:755–766.
11. Croce CM, Calin GA (2005) *Cell* 122:6–7.
12. Kluiver J, Kroesen BJ, Poppema S, van den Berg A (2006) *Leukemia* 20:1931–1936.
13. Chen CZ, Li L, Lodish HF, Bartel DP (2004) *Science* 303:83–86.
14. Fazi F, Rosa A, Fatica A, Gelmett V, De Marchis ML, Nervi C, Bozzoni I (2005) *Cell* 123:819–831.
15. Felli N, Fontana L, Pelosi E, Botta R, Bonci D, Facchiano F, Liuzzi F, Lulli V, Morsilli O, Santoro S, *et al.* (2005) *Proc Natl Acad Sci USA* 102:18081–18086.
16. Fontana L, Pelosi E, Greco P, Racanicchi S, Testa U, Liuzzi F, Croce CM, Brunetti E, Grignani F, Peschle C (2007) *Nat Cell Biol* 9:775–787.
17. Garzon R, Fabbri M, Cimmino A, Calin GA, Croce CM (2006) *Trends Mol Med* 12:580–587.
18. Kasashima K, Nakamura Y, Kozu T (2004) *Biochem Biophys Res Commun* 322:403–410.
19. Ziegler B, Testa U, Condorelli GL, Vitelli L, Valtieri M, Peschle C (1998) *Stem Cells* 16:51–73.
20. Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F, Berger R (1991) *Blood* 77:1080–1086.
21. Gallagher R, Collins S, Trujillo J, McCredie K, Ahearn M, Tsai S, Metzgar R, Aulakh G, Ting R, Ruscetti F, *et al.* (1979) *Blood* 54:713–733.
22. Hsu PW, Huang HD, Hsu SD, Lin LZ, Tsou AP, Tseng CP, Stadler PF, Washietl S, Hofacker IL (2006) *Nucleic Acids Res* 34:135–139.
23. Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P, *et al.* (2004) *Nature* 432:226–230.
24. Marinescu VD, Kohane IS, Riva A (2005) *BMC Bioinform* 6:79.
25. Okuno Y, Huang G, Rosenbauer F, Evans EK, Radomska HS, Iwasaki H, Akashi K, Moreau-Gachelin F, Li Y, *et al.* (2005) *Mol Cell Biol* 25:2832–2845.
26. Rosenbauer F, Koschmieder S, Steidl U, Tenen DG (2005) *Blood* 106:1519–1524.
27. Denti MA, Rosa A, Sthandier O, De Angelis FG, Bozzoni I (2004) *Mol Ther* 10:191–199.
28. Amendola M, Venneri MA, Biffi A, Vigna E, Naldini L (2005) *Nat Biotechnol* 23:108–116.
29. Bonci D, Cittadini A, Latronico MV, Borello U, Aycock JK, Drusco A, Innocenzi A, Follenzi A, Lavitrano M, Monti MG, *et al.* (2003) *Gene Ther* 10:630–636.
30. Chakrabarti SK, James JC, Mirmira RG (2002) *J Biol Chem* 277:136–13293.